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Genome Scanning Methods for Comparing Sequences Between Groups, with Application to HIV Vaccine Trials

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1. Introduction

The extensive genetic diversity of the human immunodeficiency virus (HIV) poses a formidable challenge to the development of an efficacious HIV vaccine (HVTN, 2006). An HIV vaccine may prevent infections with viruses genetically similar to a virus represented in the vaccine, but fail against genetically dissimilar viruses. Data on the amino acid sequences of the viruses that infect participants in preventive HIV vaccine efficacy trials can be used to assess how the efficacy of the candidate vaccine depends on genetic mismatching of exposing viruses. "Sieve analysis" methods have been developed for this purpose, which are based on comparing the genetic distances (to the vaccine sequence) of the sequences of infected vaccine recipients to the genetic distances of the sequences of infected placebo recipients (Gilbert, Self, and Ashby, 1998). Previously developed sieve analysis methods considered "low dimensional" cases in which viruses are classified exhaustively by a small number of K genotypes/phenotypes, or are ordered by K scalar summary measures of distance. However, there are many thousands of distinct HIV genotypes as defined by amino acid sequence. Consequently, the problem of identifying sequence patterns that differentiate between the two sets of infecting viruses is a high dimensional data problem, in which the number of variables (sequence positions) exceeds the number of observations (infected subjects). In a typical efficacy trial, 100-400 subjects are infected and 500-3300 sequence positions are studied.

The dataset available from an efficacy trial that we consider is the aligned HIV amino acid sequences sampled from infected vaccine and placebo recipients, with one sequence per subject. We develop techniques for "genome scanning", whereby a sliding

window is used within which the amino acids in the two aligned sequence sets are compared to the amino acid at the corresponding position in the reference sequence, and the goal is to identify "signature positions" (see Figure 1). A signature position is a position at which vaccinee sequences exhibit significantly greater divergence from the reference amino acid than placebo sequences. Identifying a signature position may suggest that amino acid changes in that position were required in order for HIV to elude the vaccine-induced immune response and hence establish infection. For example, certain N-linked glycosylation positions in the glycoprotein 120 (gp120) region of HIV appear critically important for HIV to evade neutralization (Wei et al., 2003), and the vaccine may fail to protect against viruses with certain mutant amino acids in these positions. Finding a signature position could imply the necessity to add multiple different antigens to the vaccine, with amino acid sequences that match contemporary circulating viral strains, in order for the vaccine to protect broadly. Therefore the results of genome scanning analyses can guide the design of new vaccines.

Consideration of one of the most commonly used methods for studying HIV signature positions, VESPA (Korber and Myers, 1992; http:hiv-web.lanl.govcontenthiv-dbmainpage.html), demonstrates the need for new methodology. VESPA is purely descriptive- it compares the frequency of the most common amino acid at positions between two sequence sets, without weighting the particular amino acids involved, and without using a probabilistic framework to control error rates. Our approach to the scanning analysis divides into three parts:

1. For each position, construct a two-sample test statistic that compares amino acid divergences or frequencies between the two groups;

2. Approximate the null distribution of the test statistics across the set of studied amino acid positions, and obtain position-specific p-values;

3. Apply a multiple testing adjustment procedure to the set of unadjusted p-values to infer the set of signature positions, controlling for a false positive rate.

For 1., various statistics for evaluating sequence distances have recently been proposed, based on standardized Euclidean distance and Kullback-Leibler discrepency (Wu, Hsieh, and Li, 2001), and Mahalanobis distance (Kowalski, Pagano, and DeGruttola, 2002). These metrics/discrepencies were developed in different contexts than genome scanning analysis, so their relative utility for our application is unknown. Accordingly we develop and compare test statistics based on all three of these approaches, generalized to incorporate weight functions that can make amino acid distances more immunologically relevant and thus hopefully more predictive of vaccine efficacy.

The test statistics evaluate the null hypothesis that the amino acids in the two sets of sequences have equal distributions of divergence to the reference amino acid. We also construct versions of the statistics that test for equal frequency distributions of amino acids at the position, irrespective of a reference amino acid.

For 2., we consider two approaches to approximating the null distributions. The first is a standard permutation procedure that only uses information at individual positions. The second approach, following Pan (2003), pools information across all positions and estimates the null distributions of the test statistics directly and nonparametrically. Efron (2004) also pointed out that a large number of tests presents an opportunity to estimate the null distribution directly as a novel approach to coping with high dimensional data. We apply both approaches to obtain unadjusted p-values for each of the positions, which are then subjected to a multiple comparisons adjustment procedure.

For 3., we apply the modified Bonferroni method developed by Tarone (1990) to control the family-wise type I error rate (FWER) across positions, as well as a recent modification of the original false discovery rate (FDR) procedure that also exploits the

discrete characteristics of the genetic data to increase power (Gilbert, 2005).

This article is organized as follows. Section 2 develops three new test statistics for identifying signature positions. Section 3 describes the procedures for obtaining p-values and summarizes the multiple comparisons techniques employed, and describes two slightly modified test statistics that are suitable for use with the nonparametric estimation method for deriving p-values. Section 4 compares the performance of the various methods in numerical studies, Section 5 presents an example from the first HIV vaccine efficacy trial, and Section 6 gives concluding remarks.

2. Genome Scanning Methods for Identifying Signature Positions

2.1 *Preliminaries*

The data available for genome scanning analysis are $n_1 + n_2 + 1$ aligned amino acid sequences, one from each infected trial participant (n_1 vaccine arm; n_2 placebo arm), plus a reference sequence represented in the vaccine construct, all of which are p amino acids long. The amino acids compose HIV proteins, and the analysis considers the set of positions that constitute the HIV proteins expressed by the tested vaccine. Current vaccine candidates express proteins spanning $p \sim 500 - 3300$ positions (HVTN, 2006).

For the ith position and the jth sequence in the kth group, $k = 1, 2$, we define a vector of indicators to represent the 20 amino acids possible at position i, including the possibility of a gap which may have arisen in the alignment. Specifically, let $Y_{kj}(i) = (Y_{kj}(i,1), \cdots, Y_{kj}(i,21))'$, where $Y_{kj}(i,a)$ is 1 if amino acid a is at position i and 0 otherwise, $a = 1, \cdots, 20$ ($a = 1$ represents A, Alanine; $a = 2$ represents C, Cysteine; and so on in alphabetical order), and $a = 21$ represents a gap. Similarly define $Y_r(i) = (Y_r(i,1), \cdots, Y_r(i,21))'$ for the reference sequence, and let $r(i)$ denote the amino acid at position i in the reference sequence. The vector $Y_{kj}(i)$ is a 21-nomial random variable with response probability vector $p_k(i) = (p_k(i,1), \cdots, p_k(i,21))'$. The

MLE of $p_k(i)$ is $\widehat{p}_k(i) = (\bar{Y}_k(i,1), \cdots, \bar{Y}_k(i,21))'$, where $\bar{Y}_k(i,a) = n_k^{-1} \sum_{j=1}^{n_k} Y_{kj}(i,a)$.

The biological significance of a difference in two amino acids at a position depends on the particular amino acids being compared (e.g., T vs Y). There is a vast literature on how to weight the $20\times\ 19 = 380$ different amino acid mismatches, by physico-chemical or evolutionary properties, and our methods incorporate a weight matrix to reflect such information. Specifically, let M be a 21×21 matrix with nonnegative entries, with $(a, a')^{\text{th}}$ element the weight/score summarizing dissimilarity of amino acids a and a'. For example, the nondiagonal entries of M could be taken to be those from a hydrophobicity/biochemical scoring matrix (George, Barker, and Hunt, 1990). The distance between the amino acid at position i in the j^{th} sequence of group k to the amino acid at position i in the reference sequence, $r(i)$, is the appropriate element of M, computed as $d_{kj}(i) = Y_{kj}(i)' M Y_r(i)$. The simplest matrix $M = J - I$, with J the 21 by 21 matrix of ones and I the identity matrix; with this matrix $d_{kj}(i)$ is one if the two amino acids under comparison are different and is zero if they are the same.

2.2 Two-sample Test Statistics

For each position i, test statistics are developed to evaluate $H_0(i) : p_1(i) = p_2(i)$ versus $H_1(i) : p_1(i) \neq p_2(i)$. The choice of weight matrix M determines whether the procedures test for differential amino acid divergence from the reference amino acid or for differential amino acid frequencies, irrespective of any reference. Zeros on the diagonal of M yields tests of the former type, and $M = J$ of the latter type.

For position i, set

$$\widehat{v}^2(i,a) = M(a, r(i)) \left[\frac{(n_1 - 1)}{(n-2)} \widehat{Var}(\widehat{p}_{11}(i,a) + \frac{(n_2 - 1)}{(n-2)} \widehat{Var}(\widehat{p}_{21}(i,a)) \right] M(a, r(i)).$$

Let $p^*(i)$ be the number of nonzero components in $\widehat{v}^2(i,a)$ minus one, and define

$$Z_E(i) = C_E(i) \sum_{a=1}^{21} \frac{(M(a, r(i)) \left[\widehat{p}_1(i,a) - \widehat{p}_2(i,a)\right])^2}{\{\widehat{v}(i,a) + \lambda_1\}^2} I(\widehat{v}(i,a) > 0), \qquad (1)$$

where $C_E(i) = [(n - p^*(i) - 1)/(p^*(i)(n-2))] \times [(n_1 - 1)(n_2 - 1)/(n-2)]$ and λ_1 is a nonnegative constant. Note that $M(a, r(i))\,[\widehat{p}_1(i,a) - \widehat{p}_2(i,a)] = \bar{d}_1(i,a) - \bar{d}_2(i,a)$. The constant λ_1 is added to the denominator of $Z_E(i)$ to stabilize the statistics, which can be large due to small $\widehat{v}(i,a)$. Efron et al. (2001), Tusher et al. (2001), and Guo et al. (2003) suggested adding a small positive constant to two-sample statistics in high dimensional data microarray applications, and Lönnstedt and Speed (2002) showed that the modified statistics perform better than the usual t-statistic. Following the approach of Tusher et al. (2001), we choose λ_1 to minimize the coefficient of variation of $\{Z_E(i) : i = 1, \cdots, p\}$. An alternative approach would pick λ_1 as the 90^{th} percentile of $\{\widehat{v}(i,a) : i = 1, \cdots, p; a = 1, \cdots, 21\}$ (Efron et al., 2001).

For the second test statistic, Mahalanobis' D^2 statistic for position i is given by

$$D^2(i) = (\widehat{p}_1(i) - \widehat{p}_2(i))' diag(MY_r(i))\widehat{S}^-_{\lambda_2}(i) diag(MY_r(i))(\widehat{p}_1(i) - \widehat{p}_2(i)),$$

where $\widehat{S}^-_{\lambda_2}(i)$ is the Moore-Penrose generalized inverse of $\widehat{S}_{\lambda_2}(i) \equiv \widehat{S}(i) + \lambda_2 diag(\underline{1}_{nz}(i))$, with $\widehat{S}(i) = [(n_1 - 1)\widehat{S}_1(i) + (n_2 - 1)\widehat{S}_2(i)]/(n-2)$ and λ_2 a nonnegative constant. Here $\widehat{S}_k(i) = diag(\widehat{p}_k(i)) - \widehat{p}_k(i)\widehat{p}_k(i)'$ is the multinomial MLE of $S_k(i) = diag(p_k(i)) - p_k(i)p_k(i)'$, and $\underline{1}_{nz}(i)$ is the 21-vector of indicators of whether the ath row of $\widehat{S}(i)$ is not the zero vector, $a = 1, \cdots, 21$. $S^-_{\lambda_2}(i)$ can be computed by calculating the Moore-Penrose inverse of the submatrix of $\widehat{S}_{\lambda_2}(i)$ formed by removing the zero-vector rows and columns (corresponding to amino acids never present or always present at position i), and then expanding the generalized inverse to a 21×21 matrix by re-inserting the zero-vector rows and columns. When $M = J$ and $\lambda_2 = 0$, $D^2(i)$ is the Mahalanobis statistic that has been used extensively (cf., Rao and Chakraborty, 1991).

The second test statistic, $Z_M(i)$, is defined by

$$Z_M(i) = \frac{(n - p^*(i) - 1)}{p^*(i) \times (n-2)} \frac{n_1 n_2}{n} D^2(i).$$

Similarly to $Z_E(i)$, the diagonal matrix $\lambda_2 diag(\underline{1}_{nz}(i))$ is added to $\widehat{S}(i)$ to stabilize $Z_M(i)$. The constant λ_2 is selected to minimize the coefficient of variation of the test statistic via the algorithm of Guo et al. (2003, page 1630). A potential advantage of $Z_M(i)$ compared to $Z_E(i)$ is that it accounts for the correlation structure of the multinomial response vectors, which could possibly increase statistical power.

The third statistic is based on the Kullback-Leibler discrepency, which is relatively easy to compute. For position i, let $Z_{KL}(i) =$

$$\sum_{a=1}^{21} M(a, r(i))\widehat{p}_1(i,a)\log\left\{ I(\widehat{p}_2(i,a) > 0)\frac{\widehat{p}_1(i,a)}{\widehat{p}_2(i,a)} + I(\widehat{p}_2(i,a) = 0)\frac{(\widehat{p}_1(i,a) + n_1^{-1})}{n_2} \right\}. \quad (2)$$

Wu, Hsieh, and Li (2001) suggested placing the indicator $I(\widehat{p}_2(i,a) > 0)$ in the Kullback-Leibler discrepency, which for our problem prevents $Z_{KL}(i)$ from taking infinite value.

3. Judging Statistical Significance

3.1 Permutation-based Unadjusted p-values

To judge statistical significance of the p tests, first nominal (unadjusted) position-wise p-values are computed. Since analytic p-values based on limiting distributions are not available for the test statistics (except Euclidean and Mahalanobis with $\lambda_1 = \lambda_2 = 0$), and parametric distributional assumptions may be unreliable given the underlying discreteness of the sequence data, we use a permutation procedure to determine p-values. Specifically, B data sets, each of $n = n_1 + n_2$ sequences, are generated by independently permuting the group membership labels of the whole sequences. The p-value for position i is calculated as the fraction of the test statistics computed using the B permuted datasets that equal or exceed the value of the original test statistic.

3.2 Nonparametric Estimated Null Distribution-based Unadjusted p-values

In the second (pooling) approach to computing position-specific p-values, slightly modified versions of $Z_E(i)$ and $Z_M(i)$ are needed, as described below. These modified statistics advantageously incorporate a position-specific weight $w_1(i), i = 1, \cdots, p$,

which can be used to reflect biological information. For example, positions could be weighted by their conservancy (a position is relatively conserved if most sequences contain the same amino acid at the position), since conserved positions may be more functionally or structurally important than variable positions. For exploratory analyses, where the aim is to generate hypotheses about positions that warrant further biological examination, equal weights $w_1(i) = 1$ may be recommended, because they prevent subjective biases from influencing the results, and they may be agreed upon broadly across investigators. For these reasons equal weights are used in the Example.

To develop the pooling approach, we follow Pan's (2003) clever idea for how to directly nonparametrically estimate the null distribution of hundreds of t-statistics. Assume that under all $H_0(i)$'s, the test statistics of interest $Z(i)$ have the same distribution for $i = 1, \cdots, p$. For each group of sequences separately, randomly permute the sequences into two (almost) equally-sized pieces, labeled sets $J_{k1}, J_{k2}, k = 1, 2$. Define $n_{k2} = n_{k1}$ if $n_k = 2n_{k1}$ and $n_{k2} = 2n_{k1} + 1$ otherwise, $k = 1, 2$. Modify (slightly) the test statistic $Z_E(i)$ of (1) to $Z_E^{split}(i) = w_1(i)C_E(i)\times$

$$\sum_{a=1}^{21} \frac{\{M(a, r(i)) \left[(\widehat{p}_{11}(i, a) + \widehat{p}_{12}(i, a))/2 - (\widehat{p}_{21}(i, a) + \widehat{p}_{22}(i, a))/2\right]\}^2}{\{\widehat{v}(i, a) + \lambda_1\}^2} I(\widehat{v}(i, a) > 0),$$

where $\widehat{p}_{k1}(i, a) = n_{k1}^{-1} \sum_{j=1}^{n_k} Y_{kj}(i, a) I(j \in J_{k1})$ averages the $Y_{kj}(\cdot)$ in the first permuted half of sample k and $\widehat{p}_{k2}(i, a)$ averages the $Y_{kj}(\cdot)$ in the second permuted half. The statistic $Z_E^{split}(i)$ approximately equals $Z_E(i)$, and motivates a statistic that estimates its null distribution: $z_E^{split}(i) = w_1(i)C_E(i)\times$

$$\sum_{a=1}^{21} \frac{\{M(a, r(i)) \left[(\widehat{p}_{11}(i, a) - \widehat{p}_{12}(i, a))/2 + (\widehat{p}_{21}(i, a) - \widehat{p}_{22}(i, a))/2\right]\}^2}{\{\widehat{v}(i, a) + \lambda_1\}^2} I(\widehat{v}(i, a) > 0).$$

Because the numerator of $z_E^{split}(i)$ is the sum of within-sample differences, its mean is zero. Furthermore, the denominators of $Z_E^{split}(i)$ and $z_E^{split}(i)$ are the same, and thus $z_E^{split}(i)$ can be expected to approximate the null distribution of $Z_E^{split}(i)$.

To obtain p-values, once $Z_E^{split}(i)$ is computed, each group of sequences is again separately randomly permuted into two halves, and $z_E^{split}(i)$ is computed. Based on B separate permutations $z_E^{split(b)}(i)$ is computed B times, $b = 1, \cdots, B$. For position i the p-value is then $p_i = N_i/(B \times p)$, where N_i is the number of the test statistics $z_E^{split(b)}(i')$ that equal or exceed $Z_E^{split}(i)$, pooling over $i' = 1, \cdots, p$ and $b = 1, \cdots, B$.

We use a very similar approach to estimate the null distribution of a slightly modified version of $Z_M(i)$, $Z_M^{split}(i)$, defined as

$$Z_M^{split}(i) = w_1(i)\frac{(n - p^*(i) - 1)}{p^*(i) \times (n-2)}\frac{n_1 n_2}{n}D^{2split}(i), \tag{3}$$

where $D^{2split}(i)$ is the same as $D^2(i)$ with $\widehat{p}_1(i) - \widehat{p}_2(i)$ replaced with $(\widehat{p}_{11}(i) + \widehat{p}_{12}(i))/2 - (\widehat{p}_{21}(i) + \widehat{p}_{22}(i))/2$. The null distribution of $Z_M^{split}(i)$ can be estimated via $z_M^{split}(i)$ defined as in (3) with $(\widehat{p}_{11}(i) + \widehat{p}_{12}(i))/2 - (\widehat{p}_{21}(i) + \widehat{p}_{22}(i))/2$ replaced with $(\widehat{p}_{11}(i) - \widehat{p}_{12}(i))/2 + (\widehat{p}_{21}(i) - \widehat{p}_{22}(i))/2$. P-values are then obtained in the same way as for $Z_E^{split}(i)$.

Note that the position weights $w_1(i)$ affect the p-values because the pooling method is used; weights placed in front of the non-split statistics described in Section 2 would not affect the permutation-based p-values, because they are computed marginally.

3.3 Multiple Hypothesis Testing Adjustment

Given the set of p-values, we consider four multiple comparisons adjustment procedures to determine the set of significant signature positions: standard Bonferroni, Tarone's (1990) modified Bonferroni method for discrete data, standard FDR (Benjamini and Hochberg, 1995), and Tarone-modified FDR for discrete data, which we refer to as "Tarone FDR" (Gilbert, 2005). We sketch the latter procedure in two steps: First, compute the integer K and the subset of indices R_K among the p hypotheses as described by Tarone (1990); Second, perform Benjamini and Hochberg's (1995) FDR procedure at level α on the subset of hypotheses R_K. To define K and R_K, for each $k = 1, \cdots, p$, let $m(k)$ be the number of the p positions for which $\alpha_i^* < \alpha/k$, where α_i^*

is the minimum achievable significance level for the test for the ith position, computed based on data pooled over the two groups. Then K is the smallest value of k such that $m(k) \leq k$, and R_K is the set of indices satisyfing $\alpha_l^* < \alpha/K$. Because K and R_K are calculated based only on information pooled over the vaccine and placebo groups, this procedure controls the FDR at level α. Due to the complexity of computing the α_i^* for each of the newly proposed test statistics, for the Simulations and Example the α_i^* were computed based on Fisher's exact test.

4. Simulation Study

4.1 Background

The simulation study is designed based on data from the first HIV vaccine efficacy trial (Flynn et al., 2005). Healthy HIV uninfected volunteers were randomized to receive vaccine ($N_v = 3598$) or placebo ($N_p = 1805$) and were tested for HIV infection every 6 months for 36 months. The vaccine was a recombinant envelope gp120 subunit vaccine, and was designed to prevent acquisition of HIV by inducing antibodies that could bind to neutralizing epitopes on HIV gp120 and prevent entry of HIV into host cells. The vaccine did not prevent HIV infection, with a similar rate of infection in the vaccine ($241/3598 = 6.7\%$) and placebo ($127/1805 = 7.0\%$) arms. For 336 of the 368 infected participants three HIV isolates were sampled at the time of HIV infection detection, and the amino acid sequence of gp120 was determined by direct translation of the DNA sequence for each isolate. Sequences from the same individual were highly similar, and we considered one randomly selected sequence from each subject. The 336 gp120 sequences were aligned together with the two gp120 sequences that were represented in the vaccine construct, named MN and GNE8. Since GNE8 was sampled more recently and was closer genetically to the infecting sequences, it was used as the reference sequence in all analyses. There are $n_1 = 217$ vaccine group sequences and

$n_2 = 119$ placebo group sequences, each of length $p = 581$.

For each of the five testing procedures developed above, plus Fisher's exact test for comparison, simulations were carried out to address the following questions: 1) What is the impact of the proportion of positions with a true alternative hypothesis on the performance of the procedures? 2) How much power is there to detect signature positions for vaccine efficacy trials of different sizes? 3) How do the position weights $w_1(i)$ influence size and power of the split test statistics? 4) What is the impact of the constants λ_1 and λ_2 in the performance of the Euclidean- and Mahalanobis-based procedures? To address these questions, gp120 sequences for the infected placebo group were simulated by randomly sampling with replacement $n_2 = 90$ or 180 whole sequences from the 336 sequences. Assuming an overall vaccine efficacy of 50%, sequences for the infected vaccine group were generated by sampling with replacement $n_1 = 45$ or 90 whole sequences from the 336 sequences. To create the alternative hypothesis at a given position i, we used the HIV-specific PAM matrix developed by Nickle et al. (2005) to induce stochastic evolution of the amino acids at i in the vaccinee sequences. Each nondiagonal entry of the PAM matrix corresponds to two different amino acids, and specifies the probability that either of the amino acids mutates into the other one during a certain amount of evolutionary time. We used the PAM-25 matrix, which specifies a total of 25 amino acid interchanges per 100 positions.

Question 1) was addressed by setting 1%, 10% or 25% of the positions to have true alternatives, which amounts to 6, 58, or 145 of the 581 positions. We selected the positions based on previous studies supporting that 39 of the 581 positions are important for HIV neutralization or CD4 co-receptor binding. Specifically, Wyatt et al. (1998) identified the CD4-binding positions 88, 113, 117, 256, 257, 262, 266, 368, 370, 384, 421, 427, 457, 470, 474, 475, 477, 482, 483, 484, the CD4-induced epitope positions 88,

117, 121, 207, 256, 257, 262, 370, 381, 382, 419, 420, 421, 422, 423, 427, 435, 438, 475, and positions 295, 297, 334, 386, 392, 397, which constitute a neutralization epitope defined by the monoclonal antibody 2G12. The positions, here and in the Example, are numbered using the standard HXB2 strain numbering system (Kuiken et al., 2002). In addition, Wei et al. (2003) identified three positions at which amino acid changes can sterically inhibit the accessibility of principal neutralizing epitopes on the virus surface: 245, 274, 309. These comprise 39 unique positions. For the 6 alternative positions, we selected the positions constituting the monoclonal antibody 2G12 neutralization epitope (295, 297, 334, 386, 392, 397); for the 58 alternative positions we selected the 39 key positions listed above plus 19 randomly sampled positions; and for the 145 alternative positions we used these 58 positions plus 87 more randomly sampled positions. Question 2) was addressed by repeating the simulation experiment for small (n1/n2 = 45/90 infections) and large (n1/n2 = 90/180 infections) efficacy trials. Question 3) was addressed by running simulations with $w_1(i) = I(H_0(i)\ \text{true}) + cI(H_0(i)\ \text{false})$ with c set as 2.0 or 0.5, which evaluate the split test statistics when the true alternative hypotheses are upweighted 2-fold (correctly incorporating prior knowledge) or downweighted 2-fold (incorrectly incorporating prior knowledge), respectively. Question 4) was addressed by repeating the simulations with λ_1 and λ_2 set to zero.

Amino acid substitutions were weighted equally, by setting $M = J - I$. Except for results reported at the end of Section 4.3, positions in the split statistics were weighted equally ($w_1(i) = 1$). Tests were carried out at 2-sided level $\alpha = 0.05$, using 250 permutations to approximate p-values. Empirical false positive rates, false discovery rates, and powers of the testing procedures were computed.

4.3 Simulation Results

Figure 2 shows the estimated false positive rates and FDRs using the Tarone Bon-

ferroni and Tarone FDR multiple testing adjustment methods, respectively. The tests based on $Z_E(i), Z_M(i), Z_E^{split}(i), Z_M^{split}(i)$ use $\lambda_1 = \lambda_2 = 0$, due to their superior performance as described below. All of the test procedures are conservative under family-wise error adjustment (left panels). Under FDR adjustment, when 10% or 25% of the null hypotheses are false, all of the procedures except the two Mahalanobis-based tests control the FDR below 0.05 within 2 Monte Carlo standard errors. When only 1% of the null hypotheses are false, all of the proposed procedures have estimated FDRs higher than 0.05. This occurs because in many simulation runs the null hypothesis was only rejected at 1 or 2 positions, in which case a single false rejection makes the FDR very high (e.g., 0.5 for 2 total rejections). This suggests that the FDR method should not be used unless the Tarone Bonferroni method rejects at least one null hypothesis. The tests based on $Z_E(i), Z_M(i), Z_E^{split}(i), Z_M^{split}(i)$ with $\lambda_1 > 0$ and $\lambda_2 > 0$ had very similar false positive and FDR rates.

Figure 3 shows the estimated powers of the procedures. We make several observations. First, the Kullback-Leibler and standardized Euclidean statistics are consistently most powerful. Fisher's exact statistic is third most powerful, with $Z_E^{split}(i)$ providing similar power at the larger sample size under FDR adjustment. Second, the statistics that use pooling are generally less powerful than their non-pooled counterparts, moreso for lower fractions of false null hypotheses. It appears that the pooling methods perform best when many alternative hypotheses are true (consistent with Pan, 2003).

Third, the tests based on $Z_M(i)$ and $Z_M^{split}(i)$ consistently have the lowest power. To explain the poor performance of the Mahalanobis-based statistics, note that the rank of the estimated covariance matrix $\widehat{S}(i)$ is often fairly high, which occurs because the gp120 region is highly variable. Consequently there are dozens of covariance terms to estimate, but the sample size is quite limited for doing so. Therefore, we conjecture

that the noise in covariance estimation is causing the poor performance. To support this conjecture, we repeated the simulations with all covariance estimates set to zero, in which case the Mahalanobis-based test statistics are very similar to the Euclidean-based statistics. With this modification these two approaches performed similarly.

Fourth, the tests based on $Z_E(i)$ and $Z_E^{split}(i)$ with $\lambda_1 = 0$ are consistently more powerful than the corresponding tests with $\lambda_1 > 0$ (not shown in figures). For example, for the smaller sample size and FWER adjustment, power of the $Z_E(i)$ tests with $\lambda_1 = 0$ is 0.45, 0.73, and 0.63 for 1%, 10%, and 25% of $H_0(i)$'s false, respectively, compared to 0.20, 0.59, and 0.51 for the $Z_E(i)$ tests with $\lambda_1 > 0$. Similarly, for the larger sample size and FWER adjustment, power of the $Z_E^{split}(i)$ tests with $\lambda_1 = 0$ is 0.12, 0.42, and 0.27 for 1%, 10%, and 25% of $H_0(i)$'s false, compared to 0.01, 0.27, and 0.18 for the $Z_E^{split}(i)$ tests with $\lambda_1 > 0$. This result can be explained by the fact that the sum $\sum_{a=1}^{21}$ in $Z_E(i)$ is restricted to contrasts $\widehat{p}_1(i,a) - \widehat{p}_2(i,a)$ for which the estimated variance is positive, which prevents the denominator from being very near 0. Similarly for $Z_E^{split}(i)$.

Fifth, when 1% of null hypotheses are false, the Kullback-Leibler and standardized Euclidean statistics have much greater power than the other methods. Therefore these methods are recommended for low signal-to-noise applications.

Sixth, the split statistics with true alternative positions upweighted have lower false positive/discovery rates and greater power than the equal-weighted methods; for example with $m_1/m_2 = 45/90$ and 10% of the alternative hypotheses true, the FDR of the $Z_E^{split}(i)$ tests with $\lambda_1 = 0$ is 0.0, compared to 0.032 for the unweighted tests, and power is 0.26 (0.64) under Tarone Bonferroni (Tarone FDR) compared to 0.23 (0.56) for the unweighted tests. On the other hand when the true alternative positions were downweighted, the opposite results attained: the $Z_E^{split}(i)$ tests with $\lambda_1 = 0$ give inflated FDR = 0.078 and low power 0.10 (0.21). These results provide preliminary "proof-

of-principle" that correct upweighting of positions can improve size and power of the tests based on $Z_E^{split}(i)$, but incorrect weighting can erode performance. This suggests that weighting to incorporate biological knowledge should be done with caution.

5. Example

The matrix M was taken as $J-I$, or as the HIV-specific PAM-250 matrix of Nickle et al. (2005), modified to have zeros on the diagonal and a vector of zeros added to the 21st row and 21st column. This matrix was computed based on thousands of observed among-patient mutations in HIV sequences. Because the previously available amino acid substitution matrices were built using organisms other than HIV, this PAM may yield more accurate rates of HIV amino acid interchanges. Using this PAM to weight amino acid mismatches may increase biological relevance.

With $w_1(\cdot) = 1, M = J - I$, and 10,000 permutations, Figure 4 shows the -$\log_{10}$-transformed unadjusted p-values based on the six test statistics (setting $\lambda_1 = \lambda_2 = 0$) for the 350 informative positions (i.e., those with enough diversity to possibly reject $H_0(i)$ using the Tarone Bonferroni procedure), and Figure 5 shows histograms of the test statistics. As indicated by the horizontal lines in Figure 4, after multiplicity adjustment the null hypothesis is not rejected for any positions by any of the tests, except that $Z_M^{split}(i)$ rejected one hypothesis (for position 435). The analysis was repeated for the subset of the 39 biologically-key positions described in the Simulations. Again only $Z_M^{split}(435)$ rejected, which we expect to be a false positive result due to the lack of corroboration by the other tests. Similar null results were obtained when M was set as the modified PAM matrix. The result of no signature positions can be explained by the inability of the tested vaccine to prevent HIV infection. If the vaccine has no effect on susceptibility to acquiring HIV, then the distribution of infecting sequences should be identical in the vaccine and placebo groups.

Because there was a suggestion of possible partial vaccine efficacy in non-white participants (infection rates 5.0% and 9.4% in the vaccine and placebo groups, unadjusted $p = 0.012$) and in participants with high self-reported risk behavior at baseline (infection rates 20.3% and 29.2%, unadjusted $p = 0.032$) (Flynn et al., 2005), the scanning analyses were repeated in these subgroups. No significant signatures were found, supporting that the apparent efficacy in these subgroups may not have been real.

To illustrate an application with significant signature positions, 251 gp160 subtype B HIV-1 sequences were downloaded from the Los Alamos HIV Sequence Database (Kuiken et al., 2002), 61 known to be CXCR4 co-receptor utilizing viruses and 192 known to be CCR5 co-receptor utilizing viruses. The sequences were multiply aligned, with common length $p = 857$ amino acid positions. The procedures with $M = J$ were applied to the data, to test for positions with different amino acid frequency distributions. Many significant signatures are found by all of the procedures; for example at level $\alpha = 0.05$ $Z_E(i)$ yields 25 (44) significant signature positions under Tarone Bonferroni (Tarone FDR) adjustment, and $Z_{KL}(i)$ yields 26 (51) significant signature positions. In comparison Fisher's exact test provides only 10 (22) significant signatures, demonstrating greater power of the new testing procedures.

6. Discussion

We developed and evaluated five new testing procedures for detecting signature positions that distinguish two groups of amino acid sequences. The Kullback-Leibler and standardized Euclidean test statistic (with constant λ_1 in the denominator set to 0) were most powerful and are recommended. The efficiency of the Kullback-Leibler discrepency likely derives from the fact that it is an expected log likelihood ratio, and has optimality properties closely related to those of likelihood ratio tests, as has been widely studied (Eguchi and Copas, 2002, provide a particularly clear discussion). A related

standardized Euclidean statistic was also found to perform well by Wu et al. (2001), and in our setting we conjecture that it provided greater power than the Mahalanobis-based test because it standardizes only by the variance estimates, thereby avoiding the noise introduced by estimating the entire covariance matrix nonparametrically.

An advantage of the methods developed here is that they incorporate a weight matrix specifying dissimilarity values for all pairs of different amino acids, as well as weights on amino acid positions. Therefore the techniques can flexibly incorporate biological knowledge about sequences, and can be tailored to different applications.

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Figure Legends

Figure 1. Illustration of amino acid sequence data available for genome scanning analysis, from 6 randomly selected HIV infected vaccine and placebo recipients in the VaxGen trial, aligned together with the reference HIV vaccine sequence GNE8. Each capital letter denotes an amino acid, which is a basic building block of proteins. A sequence of contiguous amino acids constitutes a protein. A - denotes a gap that arose in the alignment; gaps occur because the lengths of HIV sequences differ. The V3 loop region within the HIV protein gp120 is shown, which consists of positions 297-328 of gp160 using the HXB2 strain numbering system (Kuiken et al., 2002).

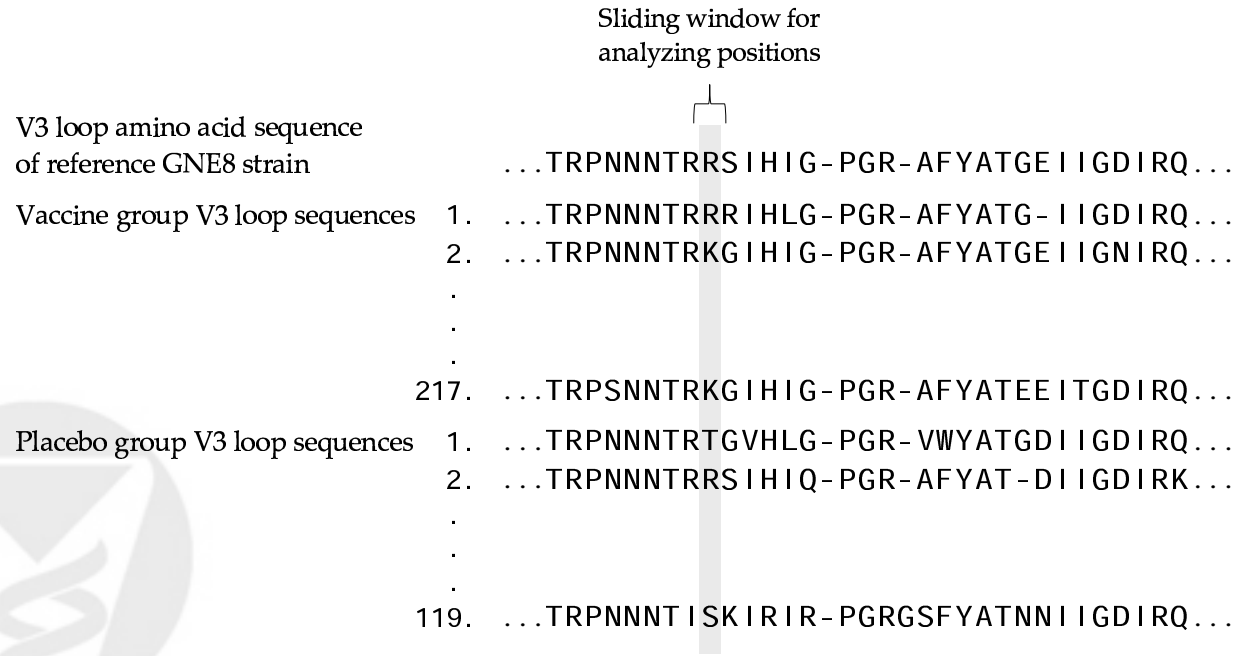
Figure 2. Based on the simulation study, panels (a) and (c) show average false positive rates for the testing procedures (with $\lambda_1 = \lambda_2 = 0$) using the Tarone Bonferroni multiple testing adjustment procedure, for $n_1/n_2 = 45/90$ infections and $n_1/n_2 = 90/180$ infections, respectively. Panels (b) and (d) show the corresponding average false discovery rates for the Tarone FDR multiple testing adjustment procedure.

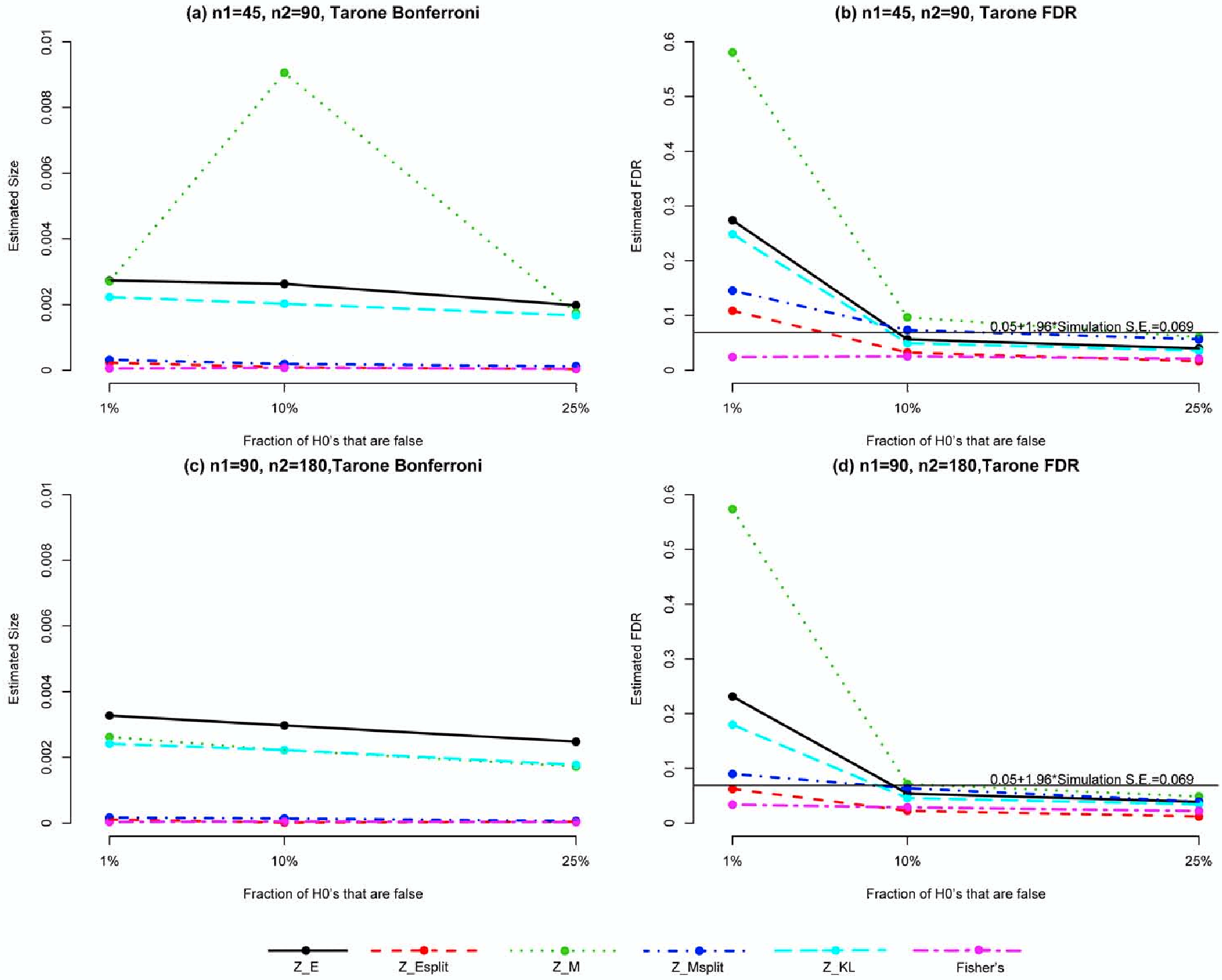
Figure 3. Based on the simulation study, panels (a) and (c) show average true positive rates (powers) for the testing procedures (with $\lambda_1 = \lambda_2 = 0$) using the Tarone Bonferroni multiple testing adjustment procedure, for $n_1/n_2 = 45/90$ infections and $n_1/n_2 = 90/180$ infections, respectively. Panels (b) and (d) show the corresponding estimated powers for the Tarone FDR multiple testing adjustment procedure.

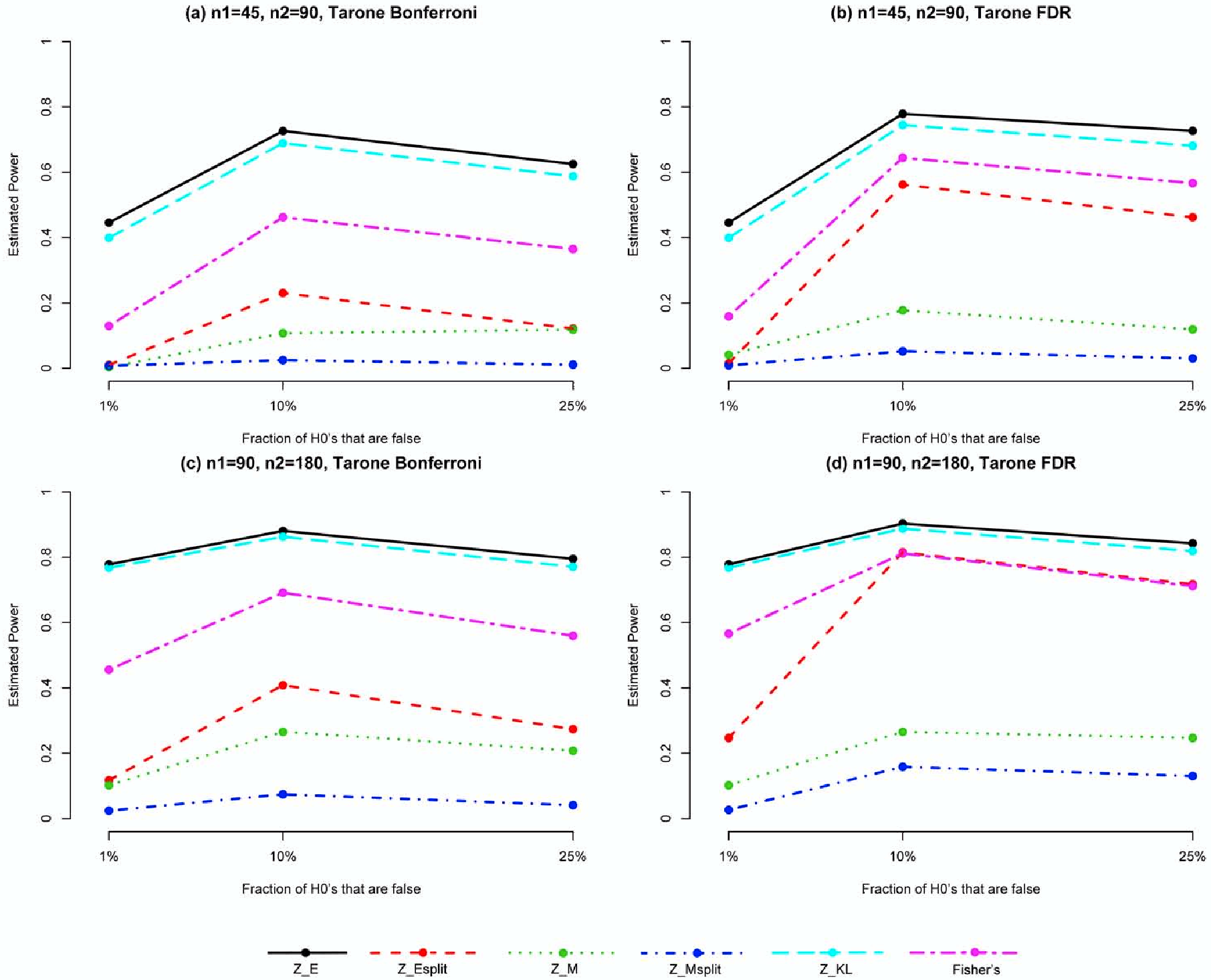
Figure 4. -$\log_{10}$ unadjusted p-values from the testing procedures (with $\lambda_1 = \lambda_2 = 0$), for the 350 informative positions among the $p = 581$ positions analyzed in the VaxGen trial, with equal weighting of all amino acid mismatches and all positions.

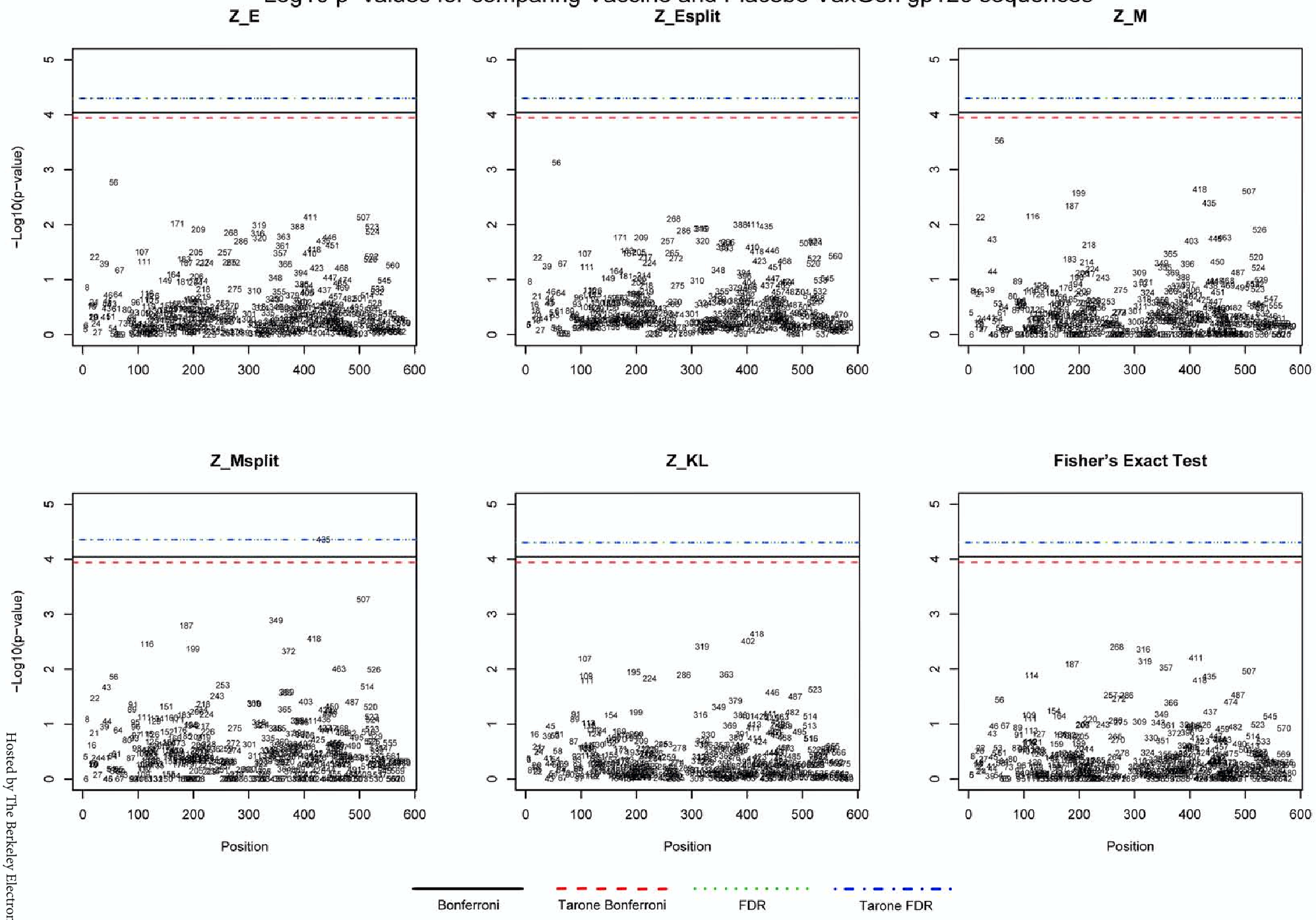
The horizontal lines represent cut-off levels of significance after adjustment for multiple testing using four different multiple testing adjustment procedures.

Figure 5. Histograms of the test statistics for the 350 informative positions among the $p = 581$ positions analyzed in the VaxGen trial, with equal weighting of all amino acid mismatches and all positions.









Histograms of Test Statistics for comparing Vaccine and Placebo VaxGen gp120 sequences